Oral neutrophil responses to acute prolonged exercise may not be representative of blood neutrophil responses.

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Running title: Blood and oral neutrophil responses to exercise

Abstract

Neutrophil numbers and function (oxidative burst) were assessed in peripheral blood and oral samples before and after prolonged exercise. Blood neutrophil count increased (~3.5-fold, $P < 0.001$) and function decreased (30 ± 19% decrease, $P = 0.005$) post-exercise. Oral neutrophil count ($P = 0.392$) and function ($P = 0.334$) were unchanged. Agreement between oral and blood neutrophil function responses to exercise was poor. These findings highlight the importance of studying neutrophils within various compartments/sample types.

Key words: exercise immunology; cycling; phagocyte; oxidative burst; immune; saliva; host defence; URTI

Introduction

It is well established that prolonged exercise causes a transient increase in the number of circulating neutrophils (neutrophilia) but their functional capacity (e.g. oxidative burst upon stimulation) is generally decreased when exercise is prolonged (> 90 min), continuous and at intensities of approximately 55% VO_2max or higher (Gleeson 2007). However, there is little research on neutrophil responses to exercise using samples other than peripheral blood. The majority of studies have not obtained neutrophils from a site local to the upper respiratory tract (URT, e.g. Davison et al. 2012; Robson et al. 1999), which may be more appropriate when concerned with URT defences. In one previous study (Müns 1994) neutrophils were obtained from nasal lavage following a 20 km run. Müns (1994) observed a significant increase in the number of neutrophils immediately after the race, which remained elevated 1 day post-race and had returned to normal by day 3. The phagocytic activity (per neutrophil) was significantly decreased immediately after the race and did not recover within 3 days. This was suggested to indicate impaired immune defences within the URT (Müns 1994). However, phagocytic capacity is considered a low-value marker and functional markers such as stimulated oxidative burst (OB) may be more valuable (Albers et al. 2005, 2013). It is disappointing, therefore, that no further exercise studies have been carried out in which other mucosal neutrophil functions are assessed. Such studies are widespread in dentistry research (e.g. Lukac et al. 2003), where associations have been observed between oral neutrophil function, salivary concentrations of neutrophil-derived antimicrobial compounds and oral health and infection risk. We have observed exercise-induced alterations in the concentration and secretion of some neutrophil-derived antimicrobial peptides (AMPs: defensins) in saliva (e.g. Human Neutrophil Peptides, HNP, 1-3), (Davison et al. 2009) but we did not determine whether they were released locally in the oral cavity or originated systemically before passing into the saliva. However, no previous study has investigated the effect of endurance exercise

on oral neutrophil functions (e.g. stimulated-OB) and this could be particularly useful to enhance understanding of the effects of exercise on URT immune function and defences. The aim of this study was to determine the effects of prolonged exercise on the number and function of neutrophils obtained from the oral cavity (and compare these with the commonly measured blood neutrophil responses).

Materials and Methods

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the University Research Ethics Committee. Written informed consent was obtained from all subjects. Subjects also completed a pre-exercise screening questionnaire before each test.

Subjects:

Nine healthy recreationally active men completed the study, although data from 2 were not included in the analysis due to oral (salivary) blood contamination. Demographic information for the remaining 7 were age 23 ± 7 years, stature 179 ± 7 cm, body mass 75.7 ± 4.4 kg, maximal oxygen uptake, $\dot{V}O_2max$, 56.9 ± 8.4 $mL \cdot min^{-1} \cdot kg^{-1}$; means ± standard deviation.

Testing protocols:

All subjects completed 3 exercise bouts; a $\dot{V}O_2max$ determination, a familiarisation trial and a main trial (each separated by 1 week). For $\dot{V}O_2max$ determination, subjects performed a continuous incremental (30 $W.min^{-1}$) test to volitional exhaustion on an electrically braked cycle ergometer (Lode Excalibur, The Netherlands) as previously described (Davison 2011). Heart rate and rating of perceived exertion (RPE) were recorded during this period using a telemetric device (Polar, Kempele, Finland) and the Borg Scale, respectively. The

familiarisation and main trials lasted 2.5 h and were identical except samples were only collected in the main trial. The trial intensity was 15% of the difference between gas exchange threshold and $\dot{V}O_2$max (15% Δ). Expired gas (10^{th}, 20^{th}, 30^{th}, 60^{th}, 90^{th}, 120^{th} min), heart rate and RPE (every 15 min) were recorded.

Subjects were all non-smokers and were required to abstain from alcohol and strenuous activity for 48 h prior to trials. They were instructed to consume 500 mL of water 2 h pre-exercise before arrival at the laboratory between 08:00 and 09:30, after an overnight fast of at least 10 h. Subjects were required to thoroughly rinse their mouth with plain water and then sit restfully for at least 10 min before collection of resting blood, saliva and oral rinse samples. Participants were then asked to consume 300 ml of a vanilla-flavoured milk-based beverage (~450 kJ, ~15 g protein, 12 g carbohydrate: lactose, and 0.2 g fat) and rest for 1 h before beginning the prolonged exercise bout. A further 50 ml of the milk-based beverage was consumed immediately prior to the exercise bout, and at 1.25 h. They were also given 2 $mL \cdot kg^{-1}$ body mass of an artificially flavoured beverage (4 parts water to 1 part 'apple and blackcurrant'-flavoured cordial) providing 48 kJ energy, 1.2 g carbohydrate and 0.4 g sodium per L of solution, every 15 min during exercise to limit dehydration. After completion of the trial, subjects were again rinsed their mouth with plain water 10 min before the saliva sample and oral rinse procedures were repeated. The post-exercise blood sample was obtained during this 10 min rest period.

Blood and saliva samples:

Samples were obtained using standard procedures and analysed immediately (blood and oral rinse) or frozen (-80°C) for later analysis (saliva) as previously described (Davison 2011). Briefly, all blood samples were obtained by venepuncture (with minimal stasis) using

Vacutainers (Becton Dickinson, Oxford, UK) and saliva was obtained by passive drool while in the seated position.

Oral rinse samples:

Oral rinse procedures were always performed after saliva samples had been obtained. Oral neutrophils were obtained with methods modified from Lukac et al. (2003) (shown to give neutrophil viability ≥ 97%). Briefly, 20 ml isotonic saline was swilled in the mouth for 2 min before expectoration. This was then concentrated 15 times by gentle centrifugation (450 × g) and replacing the supernatant with a smaller volume of buffer (HBSS: the same buffer used for dilution of blood samples, see Davison 2011, in the OB assays).

Analytical methods:

Neutrophil counts were performed using an automated haematology analyser (ABX Pentra 60C+, Horiba Medical, France).

In vitro stimulated neutrophil oxidative burst: The neutrophil PMA-stimulated OB was assessed by commercially available chemiluminescence (CL) assay, in which whole blood or oral rinse samples were first mixed with HBSS assay buffer, in accordance with the manufacturer's instructions (ABEL-04M, Knight Scientific, Plymouth, UK) as previously described (Davison 2011). PMA-stimulated and unstimulated (HBSS assay buffer in place of PMA) replicates of the same samples were monitored concurrently (30 min) to calculate the stimulated-OB (using area under the CL curves), which was then expressed per neutrophil. In line with previous work (Davison 2011; Davison et al. 2012) utilizing this assay (ABEL-04M) results were expressed relative to the appropriate pre-exercise measures.

Salivary analysis: All samples were thawed only once prior to analysis, centrifuged at 16000 × g for 2 min to pellet debris, precipitate mucins and obtain a clear supernatant. Saliva samples were screened for blood contamination using a commercially available ELISA kit (Salimetrics, USA). If saliva contamination was detected, contamination of the subsequent oral rinse sample was also assumed and data for that subject were excluded (n = 2).

Data Analysis:

Statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, Version 21.0, Armonk, NY: IBM Corp.). The pre- and post-exercise measures were compared with 2-tailed paired t-tests. All data except neutrophil function data were normally distributed (the latter were normalised with log transformation before analysis). Correlations were assessed by Pearson's correlation. Limits of agreement were calculated for the post-exercise change between sample types using the method of Bland and Altman (1986). All results are presented as mean ± standard deviation.

Results

Physiological responses to exercise (average for 2.5 h trial) were: heart rate 139 ± 13 bpm, RPE 13 ± 1, $\dot{V}O_2$ 2.4 ± 0.2 L·min^{-1} (55 ± 5% $\dot{V}O_2$max).

Blood neutrophil count increased ~3.5-fold (2.9 ± 0.8 to 10.2 ± 3.1 × 10^9 cells·L^{-1}), pre- to post-exercise ($P < 0.001$) and oral rinse neutrophil count did not change (1.2 ± 0.6 to 1.4 ± 1.0 × 10^9 cells·L^{-1}) pre- to post-exercise ($P = 0.392$). Mean oral neutrophil stimulated-OB did not change post-exercise (15 ± 37% decrease, $P = 0.334$) whereas blood neutrophil stimulated-OB significantly decreased (30 ± 19% decrease, $P = 0.005$) (Figure 1). Moreover, an actual post-exercise decrease in oral neutrophil stimulated-OB was evident in 4 of the 7

participants compared to the decrease of blood neutrophil stimulated-OB evident for all 7 participants (also, baseline absolute stimulated-OB, CL response, on a per neutrophil basis, was similar between sample types, P = 0.993). There was no correlation between the pre-to-post-exercise change (expressed as % of pre-) in neutrophil stimulated-OB (P = 0.233, r^2 = 0.269) and agreement was poor (mean difference 15.5%, ± 49.8%, 95% limits of agreement -82% to +113%). Further correlation analysis results were as follows: between the exercise-induced increase in blood neutrophil count and decrease in stimulated-OB (P = 0.189, r^2 = 0.316); exercise-induced increase in oral neutrophil count and decrease in stimulated-OB (P = 0.130, r^2 = 0.395); between sample types for baseline neutrophil count (P = 0.904, r^2 = 0.003); between sample types for baseline stimulated-OB (this measure required use of the pre-normalised CL ($RLU.s^{-1}$) data: P = 0.040, r^2 = 0.776).

*** Please insert Figure 1 near here ***

Discussion

The main findings of this study are that a significant post-exercise decrease was evident in blood neutrophil stimulated-OB (in line with much of the previous research in the exercise immunology field, e.g. Davison et al. 2012; Robson et al. 1999, and the general consensus for prolonged, i.e. > 90 min, continuous exercise, Gleeson 2007) but not in oral neutrophil stimulated-OB. However, it is possible that the study lacked power (with n = 7) to detect a mean post-exercise decrease in the oral stimulated-OB (effect sizes were small-to-medium, ~0.4, for oral *vs*. large, ~1.6, for blood neutrophil stimulated-OB). We estimate that a sample of size of ~40 would be required to detect such differences in oral neutrophil stimulated-OB, which will be a key consideration for any future research assessing oral neutrophil function. Regardless, the primary aim was to determine whether there was agreement or relationships

in the relative post-exercise changes between the two sample types (oral and blood) and our findings do suggest they differ. These findings highlight the importance of studying immune cells from locations or compartments other than peripheral blood in relation to immune defences in athletes.

A number of mechanisms have been suggested to explain the exercise-induced decreases in blood neutrophil function typically seen after prolonged exercise. This includes substrate depletion, increased stress hormones, oxidative stress and the mobilisation of cells from other (e.g. marginated and/or bone marrow) pools (Davison and Gleeson 2007; Gleeson 2007), with the latter known to be functionally immature with a lower capacity to respond to stimulation (Berkow and Dodson 1986). Whilst it is beyond the scope of this brief communication to debate each of these proposed mechanisms, the fact that neutrophil count increased in blood but not oral samples post-exercise may point towards this as a possible explanation for the lack of agreement in the exercise response of stimulated-OB between blood and oral neutrophils. However, there was no apparent relationship between the post-exercise changes in blood neutrophil counts and post-exercise changes in stimulated-OB, which does not support this hypothesis (there was also no such correlation for oral samples/neutrophils). In addition, there was no apparent correlation between sample types for the baseline neutrophil counts but there was a significant correlation for stimulated-OB. It would seem that this is not the mechanism responsible for the lack of agreement but this, and whether better agreement would be seen later into the post-exercise recovery period (possibly giving more time for blood neutrophils to migrate to oral sites), requires further investigation in larger scale studies.

The clinical relevance of exercise-induced changes in blood neutrophil functions to URT infection risk in athletes is questionable. Indeed, Albers et al. (2005, 2013) suggest that such markers have low-to-medium value in this context. It is clear that neutrophils are critical to host defence, and can affect susceptibility to such infections, highlighted for example, by the fact that patients suffering from neutrophil defects (e.g. hereditary myeloperoxidase deficiency, Kutter et al. 2000; low oxidative burst activity, Matsuzaka et al. 2008) or neutropenia (Summers et al. 2010) are known to have an increased incidence of infections compared with healthy controls. However, these neutrophil deficiencies, which are generally genetic in origin, and/or chronic in nature, will be ubiquitous across all neutrophils in the body. In healthy athletes, on the other hand, it is possible that neutrophils obtained from different sites respond differently to exercise, as demonstrated in the present study; hence a decrease in neutrophil function in one compartment (e.g. blood) may not necessarily be representative of their function in other compartments such as the oral cavity. Of note, Lukac et al. (2003) also observed a difference between oral and blood neutrophil functions in rested healthy adults. Since the URT is the main entry point for URTI-causing pathogens, the function of oral neutrophils may be of more interest regarding host defence against URTI in athletes. This is speculation at present, and this notion remains to be tested; we note that the primary aim of the present study was to compare exercise-induced changes in the functional capacity of neutrophils from blood with those from the oral cavity. We have identified a difference in neutrophil responses within these two sample types so further studies are needed to determine the relevance of the latter to URTI risk in athletes. This will require larger studies in which clinically relevant outcome markers (e.g. validated URTI assessment) are measured concurrent with oral neutrophil functions. Such studies will be particularly robust, and valuable, if designed in line with the recommendations of Albers and colleagues (2013).

237 In summary, we have demonstrated that the exercise-induced 'immunodepression' typically
238 observed in blood neutrophil stimulated-OB following prolonged (> 90 min) exercise is not
239 mirrored in oral neutrophils. This highlights the importance of studying immune cells from
240 other body compartments but further research is required to determine the importance of oral
241 neutrophil functions to host defence in athletes.
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297 **Figure captions**
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300 **Figure 1:** Oral (left) and blood (right) neutrophil oxidative burst responses.
301 **Significantly different to pre-exercise ($P < 0.01$)

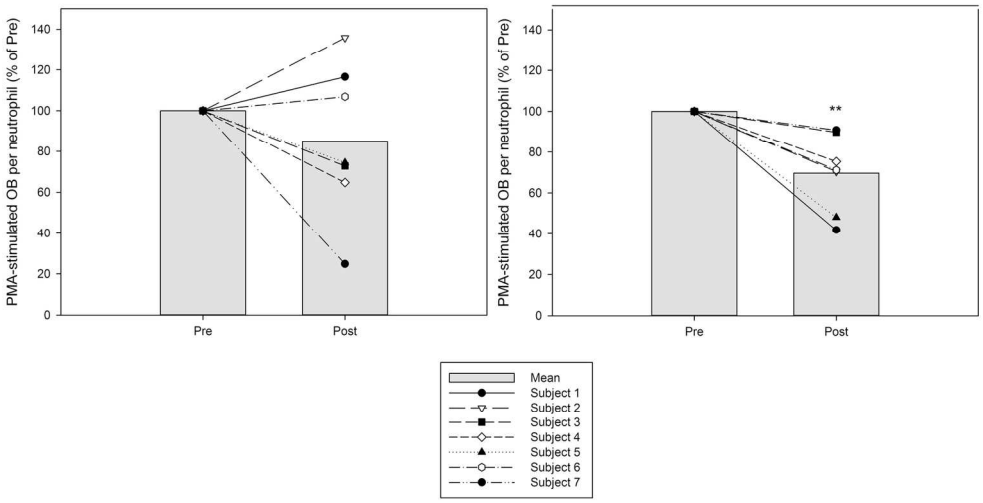

Figure 1: Oral (left) and blood (right) neutrophil oxidative burst responses.
**Significantly different to pre-exercise ($P < 0.01$)

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